Reversibility of proliferative lesions and induction of non-papillary tumors in rat urinary bladder treated with phenylethyl isothiocyanate

Satoshi Sugira1, Kumiko Ogawa1,2, Masao Hirose2, Fumitaka Takeshita1, Makoto Asamoto1 and Tomoyuki Shirai1

1Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasaki, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan and 2Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Phenylethyl isothiocyanate (PEITC), generally thought to be a chemopreventive agent for various kinds of genotoxic carcinogens, has been found to induce rat urinary bladder carcinomas in our laboratory. To cast light on the underlying mechanisms, the reversibility of urothelial proliferative lesions and the frequencies of H-ras and p53 mutations in the induced rat urinary bladder tumors were investigated. F344 male rats were given diet containing 0.1% PEITC for 48 weeks and then killed, or for 32 weeks and then returned to normal diet without supplement for 1, 3, 7 days or 16 weeks before death. At 7 days after withdrawal of PEITC treatment, carcinomas were observed in only two of 24 rats but a high incidence of dysplasias was evident. Furthermore, 16 weeks after withdrawal, seven of 12 (58.3%) rats had carcinomas. In addition, carcinomas were induced in 11 of 12 (91.7%) rats continuously receiving PEITC for 48 weeks. Most of the carcinomas were characterized as of non-papillary transitional cell type with occasional squamous cell differentiation and/or glandular components. Bromodeoxyuridine labeling indices (LIs) were increased by PEITC administration even in normal-looking epithelium. After withdrawal of treatment, LIs in simple and papillary or nodular (PN) hyperplasias were markedly decreased and these lesions gradually disappeared, while values for dysplasias and carcinomas, which persisted, were only slightly decreased. A silent point mutation was found in H-ras in one of 12 tumor samples (8.3%), whereas seven (58.3%) had mutations in p53. These results indicate that PEITC itself is a carcinogen for the rat urinary bladder, and that while the simple and PN hyperplasia induced by PEITC are reversible, dysplasia is irreversible with the potential to give rise to non-papillary carcinomas with frequent p53 mutations.

Introduction

Phenylethyl isothiocyanate (PEITC) is a natural constituent of cruciferous vegetables such as cabbages, cauliflower, Brussels sprouts and watercress (1), and mean daily intake of glucosinu- riin, a glucosinolate of PEITC, from swede-turnips in UK, is reported to be 0.8 mg/person/day (0.3 mg/person/day as PEITC) (2). The compound is known as a natural chemopreventive agent capable of potently inhibiting carcinogenesis by various chemicals. For example, it is reported to decrease 7,12-dimethylbenz[a]anthracene-induced mammary tumor development in female Sprague–Dawley rats (when given at 0.55% in the diet) (3), N-nitrosobenzylmethane-induced esophageal carcinogenesis in male F344 rats (0.005–0.08% in the diet) (4–6), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in F344 rats (0.04% in diet) and A/J mice (0.8 or 4 mg/day by intragastric tube) (7,8), when given to the animals mostly during or prior to carcinogen exposure. It may modify carcinogenesis by influencing cytochrome P-450s, which metabolize carcinogens into reactive species (9,10), or by inhibiting microsome-mediated DNA methylation (11), or by inducing phase II enzymes, which detoxify carcinogens (12). In 1998, however, we demonstrated that continuous oral administration of 0.1% PEITC for 24 weeks after carcinogen exposure enhanced urinary bladder carcinogenesis in a multi-organ carcinogenesis model (13). Subsequently, PEITC clearly enhanced the rat urinary bladder carcinogenesis (14), with dose-dependence when rats were pre-treated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) (15). It was also found that 0.1% PEITC itself induced urinary bladder carcinomas in 33% of animals with 32 weeks administration (14), suggesting possession of carcinogenic potential. However, this conclusion must remain provisional until mechanisms of enhancement of carcinogenesis or carcinogenic potential of PEITC are fully elucidated.

Genotoxicity of PEITC has been shown in an SV40-transformed Indian Muntjac cell line (16) and in a Chinese hamster ovary cell line (17), as evidenced by chromosomal aberration. PEITC-genotoxicity was also recently demonstrated in the Salmonella/microosomal assay with TA 98 and TA 100, the differential DNA repair assay with Escherichia coli and the micronucleus induction assay with human derived HepG2 cells (18). Therefore, a contribution of genotoxicity to the induction of bladder tumors by PEITC cannot be ruled out. When proliferative lesions associated with genotoxic and non-genotoxic compounds are compared, the former tended not to regress, in contrast to the case with the latter (19,20). In general, after multiple steps and accumulation of genetic alterations in oncogenes and/or tumor suppressor genes, carcinomas develop as irreversible lesions. p53 is one of the most commonly altered tumor suppressor genes in various cancers. Diverse degrees of its mutations have been reported in human (21–24) and chemically induced rat (25–27) urinary bladder carcinomas. For the better understanding of urinary bladder carcinogenesis, analysis of whether PEITC-induced bladder cancers are associated with p53 mutations is thus clearly warranted.

To cast light on the mechanisms of PEITC urinary bladder carcinogenesis in rats, the present analysis of reversibility of urothelial proliferative lesions and the presence of p53 and H-
ras mutations in cancers induced in male F344 rats was conducted.

Materials and methods

Animals and chemicals
Five-week-old male F344 rats were obtained from Charles River Japan Inc., Atsugi, Japan, and maintained in the Experimental Animal Sciences Center, Nagoya City University Graduate School of Medical Sciences. They were randomly distributed three animals per cage on hardwood chip bedding in an environment-controlled room, at 23 ± 2°C and 50 ± 5% humidity with a 12 h light and dark cycle.

PEITC (purity >98%) was purchased from Tokyo Kasei Kogyo, Tokyo, Japan. To prepare a 0.1% PEITC diet, chemical was dissolved in corn oil (10 g/kg diet; 1%), and the solution was mixed with basal diet (Oriental MF; Oriental Yeast Co., Tokyo, Japan) using a cake mixer. For the control diet, 1% corn oil alone was admixed. The diets were prepared once per 2 weeks and stored at 4°C in the dark before use, being replaced twice a week. Food and tap water were available ad libitum. The use of animals in this study followed the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Medical School and was approved by the Institutional Animal Care and Use Committee.

Animal experimentation
The experimental protocol is shown in Figure 1. At the age of 6 weeks, rats were randomized into three groups, consisting of 12, 36 and 18 rats, respectively. Animals in Group 1 were given 0.1% PEITC diet for 48 weeks and then killed. Group 2 rats received 0.1% diet for 32 weeks and then returned to control diet. Subgroups were killed at the end of 32 weeks, and 1, 3, 7 days and 16 weeks thereafter. Group 3 was maintained on control diet throughout the experiment as a control group and animals were killed at week 32 and at week 48. 0.1% PEITC in diet; ▲, control diet; ▲, death 1 h after intraperitoneal injection of 100 mg/kg body wt of BrdU.

Fig. 1. Experimental protocol. Animals in Group 1 were given 0.1% PEITC in diet for 48 weeks and then were killed. Group 2 were given 0.1% diet for 32 weeks and the diet changed into control diet (basal diet containing 1% corn oil without PEITC). Animals in this group were killed at the end of 32 weeks, and 1, 3, 7 days and 16 weeks after changing to control diet. Group 3 was maintained with control diet throughout the experiment as a control group and animals were killed at week 32 and at week 48. 0.1% PEITC in diet; ▲, control diet; ▲, death 1 h after intraperitoneal injection of 100 mg/kg body wt of BrdU.

Results
PEITC at the dose given suppressed body weight gain ~6–8% throughout the experiment, but after the withdrawal of PEITC administration at week 32, body weight gain slightly recovered (data not shown). Figure 2 illustrates the relative urinary bladder weights at the various time points of death in the three groups. Treatment with PEITC caused a significant increase and while discontinuation at week 32 resulted in a gradual decrease, in a duration-dependent manner, even after 16 weeks values were still significantly higher than normal.

Macroscopically, urinary bladders continuously treated with PEITC for 32 or 48 weeks became less translucent and thicker than control. Especially, 48 weeks administration caused roughening of the surface of the urinary bladder. Protrusion of tumors was not observed. The bladders, 16 weeks after withdrawal of PEITC treatment had discoid and irregular foci of thickening surrounded by thin mucosa.

Proliferative lesions of the urinary bladder were classified into simple hyperplasia, papillary or nodular (PN) hyperplasia, dysplasia and carcinomas, including transitional cell carcinomas with squamous cell and adenocarcinoma components (Table II). Usually PEITC-induced tumors showed downward
and nodular growth. In this experiment, no papillomas comparable with those induced by urinary bladder carcinogens such as BBN (28,29), N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) (30,31), or the combination of 3,2'-dimethyl-4-aminobiphenyl and butylated hydroxyanisole (32) were found. Dysplasia was characterized as slightly thickened urothelium with moderate cellular atypia, and frequent mitoses (Figure 3A and C).

Carcinomas were not found in rats killed 0, 1 or 3 days after withdrawal of PEITC-administration, but were noted in two of six rats killed after 7 days and in seven out of 12 after 16 weeks (Figure 3B). The latter incidence was statistically significant as compared with the negative control value. Continuous administration of PEITC for 48 weeks induced bladder carcinomas in 92% of the animals (Figure 3C).

There were no changes in body weight of the rats during the experiment. Bladder weights of all groups of rats, which were approximately the same, showed a gradual decrease after withdrawal of PEITC insult. However, the LIs of hyperplasias and normal-looking epithelium dramatically dropped after the withdrawal of PEITC, whereas those of dysplasias and carcinomas demonstrated only limited reduction. Apoptotic bodies were not observed in the urothelium during the process of loss of hyperplastic lesions. No histopathological changes were noted in the liver, kidneys or ureters of any of the groups of rats.

The results of mutation analysis of H-ras and p53 genes in PEITC-treated rats were almost the same as those of simple hyperplasias and tended to be higher than in the control group. However, the LIs of hyperplasias and normal-looking epithelium dramatically dropped after the withdrawal of PEITC, whereas those of dysplasias and carcinomas demonstrated only limited reduction. Apoptotic bodies were not observed in the urothelium during the process of loss of hyperplastic lesions. No histopathological changes were noted in the liver, kidneys or ureters of any of the groups of rats.

The results of mutation analysis of H-ras and p53 genes in PEITC-induced urinary bladder carcinomas are summarized in Table IV. PCR and gel-electrophoresis were performed at least twice for each sample and loci and the mutations were determined when alteration was confirmed by the second test. A silent point mutation, ATC (Ile) to ATT (Ile), was found in codon 55 of H-ras exon 2 in one out of 12 tumor samples (8.3%). Seven out of 12 samples (58.3%) had mutations in p53, four in exon 5 and four in exon 7 (Figure 5). Double mutations were found in one sample. Four of eight mutations in p53 were of silent point type. Five of nine mutations, including that found in H-ras, were C to T transitions.
Fig. 3. Histological features of the bladder lesions stained with hematoxylin and eosin (A–C) and immunostaining for BrdU (D–F) observed in 32 weeks PEITC-administration (A,D) 16 weeks after withdrawal of PEITC-administration (B,E), and 48 weeks continuous administration (C,F). Typical lesions corresponding with dysplasia and carcinoma were indicated by opened arrowhead ▼ and closed arrow ◀, respectively. High LIIs were seen in all lesions including simple HP, dysplasia, carcinoma during PEITC-administration (A, C, D, F). Carcinoma remained, but relatively large lesions returned to normal-looking mucosa with low LI (B,E). Most of the urothelium was covered with hyperplasia and dysplasia, and carcinoma with high LI (C,F).

Discussion

PEITC has been regarded as a promising chemopreventive agent, as it potently inhibits carcinogenesis in the mammary glands, forestomach, esophagus and lung, mostly when given prior to or during exposure to carcinogen (3–6,8). Recently, it was reported that in the post-exposure phase dietary administration of N-acetylcysteine conjugates of PEITC (at a dose of 0.98%, which is equal to 0.2% PEITC) also inhibited the development of benzo[a]pyrene-induced lung tumor in A/J mice (33). However, the present study clearly demonstrated continuous administration of 0.1% PEITC to induce urinary bladder carcinomas, in line with our previous studies (13,14) and the findings of Dunnick et al. (34) with allyl isothiocyanate, another isothiocyanate compound carcinogenic to the rat urinary bladder.

In general, reversible proliferative lesions do not feature multiple genetic alterations. Genotoxic compounds tend to induce not only reversible proliferative lesions (35) but also irreversiblepreneoplastic lesions from an earlier stage of carcinogenesis as compared with their non-genotoxic counterparts (20). Reversibility of lesions on withdrawal of the inducing insult may therefore provide clues as to genotoxicity as well as the pathway from preneoplastic lesions to carcinoma. In the present study, irreversible dysplastic mucosal changes were observed with 32 weeks administration of PEITC. Within this period, genetic changes sufficient for carcinogenesis were presumably induced in the urothelium.

Early lesions induced by PEITC could be readily divided into two groups, one hyperplastic with little atypia, and the
Table III. BrdU LI’s of bladder proliferative lesions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal (%)</th>
<th>Hyperplasia (%)</th>
<th>Dysplasia (%)</th>
<th>Carcinoma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Simple PN</td>
<td>32 weeks</td>
<td>32 weeks + Basal 1 day</td>
<td>32 weeks + Basal 3 days</td>
</tr>
<tr>
<td>PEITC 32 weeks</td>
<td>(6)</td>
<td>1.60 ± 0.70</td>
<td>0.34 ± 0.39</td>
<td>0.14 ± 0.12</td>
</tr>
<tr>
<td>PEITC 32 weeks + Basal 1 day</td>
<td>(6)</td>
<td>1.5 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>PEITC 32 weeks + Basal 3 days</td>
<td>(4)</td>
<td>3.6 ± 0.3</td>
<td>0.7 ± 0.3</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>PEITC 32 weeks + Basal 7 days</td>
<td>(5)</td>
<td>7.3 ± 3.4</td>
<td>5.3 ± 3.7</td>
<td>5.8 ± 1.6</td>
</tr>
<tr>
<td>PEITC 32 weeks + Basal 16 weeks</td>
<td>(12)</td>
<td>10.0 ± 0.7</td>
<td>6.5 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>Control (Basal 32 weeks)</td>
<td>(6)</td>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Control (Basal 48 weeks)</td>
<td>(12)</td>
<td>5.4 ± 3.0</td>
<td>3.2 ± 3.0</td>
<td>3.2 ± 3.0</td>
</tr>
</tbody>
</table>

( ), Effective number of rats for each lesion. All values are mean ± SD.

\( ^{ab}p < 0.05, 0.001 \) versus PEITC 32 weeks group.

\( ^{a}P < 0.001 \) versus corresponding control group.

Table IV. H-ras and p53 mutations detected by SSCP analysis

<table>
<thead>
<tr>
<th>Case</th>
<th>Lesion</th>
<th>H-ras</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exon 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>codon170:GTC(Val)→GTT(Val)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Codon55:ATC(Ile)→ATT(Ile)</td>
<td>codon294:TGC(Cys)→TAC(Tyr)</td>
</tr>
<tr>
<td>202</td>
<td>a Ca &gt; Dys sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>205</td>
<td>a Ca &lt; PN m</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b Ca sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b Ca sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>206</td>
<td>Ca sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>207</td>
<td>Ca &lt; PN m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>208</td>
<td>a Ca sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b Ca &gt; Dys sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>209</td>
<td>a Ca &gt; Dys sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a Ca &gt; Dys sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>a Ca sm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b Ca &gt; PN sm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ca, carcinoma; Dys, dysplasia; PN, papillary or nodular hyperplasia; m, mucosal growth; sm, submucosal growth.

Fig. 4. Ratio of bladder proliferative lesions of epithelium in each points. Whole urothelium in control groups (32 and 48 weeks) were occupied by normal epithelium. Carcinoma ■; dysplasia □; PN hyperplasia ▲; simple hyperplasia □; normal-looking □. ***, ***. \( p < 0.01, 0.001 \) versus PEITC 32 weeks group.

other dysplastic with many mitoses. The former disappeared after withdrawal of PEITC treatment in a time-dependent manner whereas the latter appeared to rather continuously progress. Cell proliferative activity as reflected in the BrdU LI was also high in dysplasia during PEITC administration. Moreover, at 16 weeks after withdrawal of insult, the LI had decreased to one of 10 in hyperplastic lesions, but only a little more than half in dysplasia. The data suggest autonomy of the latter in line with a histogenetic role in development of malignancies.

Regarding possible mechanisms of carcinogenic action,
in vitro formation of reactive oxygen intermediates in rat hepatocytes by a PEITC-related compound, benzyl isothiocyanate (BITC), has been demonstrated (36). In vivo, PEITC and BITC have potent cytotoxicity, and induce cell proliferation of rat urinary epithelium in the early phase of administration (unpublished data). Furthermore, evidence of genotoxicity of PEITC itself has been accumulating from in vitro studies (16–18). Especially isothiocyanates, including allyl isothiocyanate, BITC and PEITC, have been reported to cause Cu(II)-mediated DNA cleavage, frequently at thymine and cytosine in the DNA fragments from human p53 and c-Ha-ras, and formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in HL-60 cells (37). In the present investigation, while only one of the PEITC-induced carcinomas had a point mutation (8.3%) in H-ras, such genetic alteration was frequent (58.3%) in the p53 gene. Although some carcinomas analyzed in this study were found to invade the submucosa, no association was evident with the presence of a p53 mutation and invasive growth was noted. Frequencies of p53 mutations in chemically induced rat bladder carcinomas have varied among studies. FANFT-induced carcinomas were found to have a low frequency (<7.4%) (25) compared with those caused by BBN (60 and 57%) (26,27). In our series the C to T transition (four out of eight) was the most common type. It is also found in human bladder cancers at high frequency (23) and cytosine has been considered a major target of DNA cleavage caused by isothiocyanates (37).

In human bladder carcinomas, two types of growth are observed. One is the papillary non-invasive type, which is also typical of most chemically induced rat bladder carcinomas, observed with genotoxic BBN (28,29) and FANFT (31,38), as well as the non-genotoxic uracil (39). The other is the non-papillary invasive type, which accounts for most mouse bladder cancers (29,30,40). Two molecular pathways corresponding to the genesis of these two types have been proposed by Spruck et al. (24). In their study, loss of heterozygosity of chromosome 9, where $p16^{ink4b}$ or $p19^{ARF}$ is located, was observed in 24 of 70 (34%) papillary transitional cell carcinomas (Ta tumors) but was present in only three of 24 (12%) CIS and dysplasia lesions. In contrast, only one of 36 (3%) Ta tumors had a $p53$ mutation, compared with 15 of 23 (65%) CIS and dysplasias and 25 of 49 (51%) muscle invasive tumors. Similar frequencies of $p53$ mutations in human invasive bladder cancers have in fact been described in several reports (21,22). The macroscopic as well as microscopic features of PEITC-induced rat urinary bladder carcinoma did not include papillary growth, which is mostly observed in rat bladder treated with other bladder carcinogens, but rather appeared as diffuse or nodularly thickening of urothelium with downgrowth, growth with mouse bladder carcinomas. Mutations in the $p53$ gene were frequently detected in this experiment but most of them were silent with no obvious hot spots or signature. These facts suggest that loss of functional $p53$ may not be required for the non-papillary growth of urothelium in this model.

In conclusion, PEITC is a rat urinary bladder carcinogen. While some of the early proliferative lesions induced by PEITC are reversible, dysplasia persists and has the potential to give rise to non-papillary carcinomas, indicating genetic changes for cancers are already induced by week 32. Also, it is suggested that PEITC-induced rat urinary bladder carcinoma develop through different pathways compared with their counterparts due to well-known bladder carcinogens such as BBN, FANFT and uracil.

Acknowledgements

This work was supported in part by Grants-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare, a Grant-in-Aid from the Ministry of Health, Labour and Welfare for the Second-Term Comprehensive 10-Year Strategy for Cancer Control, Japan, and a grant from the Society for Promotion of Toxicological Pathology of Nagoya, Japan.

References


Reversibility of PEITC-induced rat bladder lesions


Received November 13, 2002; revised November 28, 2002; accepted December 5, 2002.